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Associations between child abuse, mental health and epigenetic modifications in the *POMC* gene: A study with children in Tanzania

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Abstract

Child abuse is associated with a number of emotional and behavioral problems. Nevertheless, it has been argued that these adverse consequences may not hold for societies in which many of the specific acts of abuse are culturally normed. Epigenetic modifications in the genes of the hypothalamic pituitary adrenal (HPA) axis may provide a potential mechanism translating abuse into altered gene expression, which subsequently results in behavioral changes. Our investigation took place in Tanzania - a society in which many forms of abuse are commonly employed as disciplinary methods. We included 35 children with high exposure and compared them to 25 children with low exposure. Extreme group comparisons revealed that children with high exposure reported more mental health problems. Child abuse was associated with differential methylation in the *POMC* gene, measured both in saliva and in blood. Hierarchical clustering based on the methylation of *POMC* found two distinct clusters. These corresponded with children's self-reported abuse, with two-thirds of the children allocated into their respective group. Our results emphasize the consequences of child abuse based on both molecular and behavioral grounds, providing further evidence that acts of abuse affect children, even when culturally acceptable. Furthermore, on a molecular level our findings strengthen the credibility of children's self-reports.

Keywords: *child abuse, DNA methylation, HPA axis, POMC gene, mental health*

Introduction

Child abuse is commonly defined as any act of commission by a parent or any other caregiver that results in harm, potential for harm, or threat of harm to a child (Leeb, Paulozzi, Melanson, Simon, & Arias, 2008). Child abuse may result in emotional and behavioral problems that begin in childhood and can persist throughout adolescence and adulthood (Carr, Martins, Stingel, Lemgruber, & Juruena, 2013). For example, child abuse increases the risk of developing depression, anxiety disorders, posttraumatic stress disorder (PTSD), substance abuse, reduced self-esteem, suicidal behavior, conduct disorder, and aggressive or delinquent behavior (Catani, Jacob, Schauer, Kohila, & Neuner, 2008; Dube et al., 2003; Hermenau, Hecker, Elbert, & Ruf-Leuschner, 2014; Sugaya et al., 2012), as confirmed by numerous longitudinal studies (Kaplan et al., 1998; Widom, DuMont, & Czaja, 2007). Most abused children have been exposed to multiple forms of abuse, and the greater the number of different forms of abuse, the higher the likelihood of subsequent psychopathologies (Teicher, Samson, Polcari, & Mcgreenery, 2006). Furthermore, abused individuals with a psychiatric disorder are characterized by earlier onset of disease, increased symptom severity, increased comorbidity, increased risk of suicide, poorer treatment response and shorter interval before recurrence than individuals with the same diagnoses who were not abused (Harkness, Bagby, & Kennedy, 2012; Nanni, Uher, & Danese, 2012; Teicher & Samson, 2013). Finally, child abuse is a major burden not only upon the affected individual but also upon the society at large due to the high costs associated with the utilization of healthcare, educational, welfare, and law enforcement services (Fang, Brown, Florence, & Mercy, 2012).

It has been argued that the aforementioned adverse consequences may not hold for societies or communities in which many of the specific acts of child abuse are culturally normed and highly prevalent. In other words, abused individuals in communities that deem such practices to be socially acceptable and legal would find the effects to be less harmful than those living in societies in which such practices are unacceptable or illegal. Lansford et

al. (2005) empirically tested this idea in six countries. They found that more frequent corporal punishment is related to more aggression and more anxiety in all six countries. However, the strength of the relation did vary by the perceived normativeness across countries. Many other studies demonstrated detrimental consequences for the psychological well-being and development of abused children, regardless of whether or not the surrounding society deems such practices acceptable (Ani & Grantham-McGregor, 1998; Hecker, Hermenau, Isele, & Elbert, 2014; Hermenau et al., 2011).

There are many countries in which many of the acts constituting child abuse are legal and socially accepted. In Tanzania, for example, a national survey with a representative sample of more than 3700 youths revealed that the great majority (almost 75%) of both girls and boys had experienced physical abuse and more than one quarter faced emotional abuse prior to the age of 18 (UNICEF, 2011). Concordantly, we and others reported the use of harmful physical acts and psychological tactics on behalf of caregivers towards children to be highly prevalent in Tanzanian families and schools (Feinstein & Mwahombela, 2010; Hecker et al., 2014). In April 2013, the Tanzanian Government reportedly confirmed that the use of corporal punishment in public schools persists (Tanzania Daily News, 2013). Given such high prevalence of child abuse, it is vital for both individuals and societies to have a better understanding of the potential effects of abuse. In particular, whether the negative consequences of physical and emotional abuse of children are diminished in societies where such acts are legal and socially accepted.

Most studies on mental health problems have been conducted in Western samples. However, findings from DR Congo, Ethiopia and Nigeria have shown that various mental health problems such as anxiety disorders, affective disorders and hyperactivity are also common phenomena in Sub-Saharan Africa (Adelekan, Ndom, Ekpo, & Oluboka, 1999; Kashala, Elgen, Sommerfelt, & Tylleskar, 2005). Adelekan et al. (1999) indicated a prevalence rate of internalizing problems of 7.3% and of externalizing problems of 8% in a

representative sample from Nigeria. Kashala et al. (2005) compared their findings in a study with a representative sample in DR Congo (Goodman, Meltzer, & Bailey, 1998) with prior findings from Great Britain. They found that the mean scores on all subscales of the Strength and Difficulties Questionnaire (SDQ) were significantly higher than the British mean scores of a comparable sample. Hence, Cortina, Sodha, Fazel, and Ramchandani (2012) concluded that child and adolescent mental health problems are also common in Sub-Saharan Africa.

Child abuse and the HPA axis

The hypothalamic pituitary adrenal (HPA) axis, when functioning properly, helps us to deal with crises. It describes a set of interactions between the hypothalamus, the pituitary and the adrenal gland, which results in the release of its effector cortisol (Chrousos & Gold, 1992; de Kloet, Joëls, & Holsboer, 2005). Upon stress perception, cortiocotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) are released from the hypothalamic paraventricular nucleus to activate the synthesis of pro-opiomelanocortin (POMC) in the anterior pituitary. POMC is processed into several peptides including adrenocorticotropic hormone (ACTH). Finally, ACTH is released into the blood stream and triggers the secretion of cortisol from the adrenal cortex. At each organizational level, the HPA-axis is tightly regulated by negative feedback loops meditated by glucocorticoid receptors. After binding their ligand, cortisol, glucocorticoid receptors dampen HPA-axis activity.

Child abuse is translated into negative long-term mental health outcomes via the HPA axis. It plays a central role, as it is tuned to experiences occurring early in life, making it highly susceptible to early childhood adversities (Heim & Nemeroff, 2001). For example, adults with a history of childhood maltreatment displayed altered ACTH- and cortisol-responses following exposure to an acute stressor (Carpenter et al., 2007; Heim et al., 2000). HPA-axis dysregulation is a key feature of a range of psychopathological symptoms (Chrousos & Gold, 1992; de Kloet et al., 2005). Both human and animal studies show that

unremitting threat or stress weakens the immune response, increases abdominal fat, mental ill-health, and depression via alterations of HPA functioning (McEwen & Lasley, 2002). HPA axis function, and with it behavioral changes, may be stably altered through aberrant epigenetic modifications, established as the result of child abuse.

Epigenetic modifications of HPA axis genes

Of the various and complex mechanisms leading to epigenetic modification, DNA methylation is currently being studied most extensively. In humans, the relationship between early life adversities and the methylation of the glucocorticoid receptor *(GR)* has been extensively studied. *GR* promoter methylation is associated with both child abuse and psychopathology (Dammann et al., 2011; Hompes et al., 2013; Labonte, Azoulay, Yerko, Turecki, & Brunet, 2014; McGowan et al., 2009). Suicide victims with a history of childhood abuse displayed increased *GR* methylation in brain tissue (Labonte et al., 2012; McGowan et al., 2009). Higher *GR* methylation in peripheral blood mononuclear cells has been observed in patients suffering from borderline personality disorder, i.e., in individuals who have usually been exposed to severe forms of abuse during development. Disruption or lack of adequate nurturing, as measured by child maltreatment and inadequate parental care, was also associated with increased *GR* promoter methylation (Perroud et al., 2011; Tyrka, Price, Marsit, Walters, & Carpenter, 2012). In addition, epigenetic changes in the *proopiomelanocortin* (*POMC*) gene may promote HPA axis dysfunction. Recent studies suggest epigenetic programming of *POMC* operates through nutritional cues, such as being underweight (Ehrlich et al., 2010), while other research suggests an association with alcohol abuse and dependence via increased craving (Muschler et al., 2010). Animal models demonstrate epigenetic programming of additional HPA-axis genes such as *CRH* (Mueller & Bale, 2008) and *AVP* (Murgatroyd et al., 2009). Thus, current research has highlighted

epigenetic modifications in genes associated with the HPA axis as being a possible driving force producing child abuse-induced disorders.

In the present study we investigated associations of child abuse with both the phenotype and the methylation status of genes related to the HPA axis in Tanzanian children. We limited our analyses of DNA methylation to the genes coding for the main components of the HPA axis. That is, the genes coding for arginine-vasopressin (*AVP*), corticotrophin-releasing hormone (*CRH*) and pro-opiomelanocortion (*POMC*), from which adrenocorticotropic hormone (ACTH) is cleaved. In addition we included the gene encoding the glucocorticoid receptor (*NR3C1*), as several studies demonstrated its methylation status as being predictive for childhood abuse (Labonte et al., 2012; McGowan et al., 2009; Perroud et al., 2011; Tyrka et al., 2012). We hypothesized that (a) exposed children report more emotional and behavioral problems and (b) display altered epigenetic modifications in the genes related to HPA axis functioning.

Methods

Procedure

In the context of a larger research project, a team of Tanzanian and German psychologists conducted structured interviews with a sample of Tanzanian school children (N = 409). Interviewers were taught in interview skills during a two-week training session. Furthermore, the Tanzanian interviewers were trained to translate from English to Swahili and back in order to assist the German researchers. All instruments were translated in written form to Swahili. A valid and accurate translation into English was ensured through the use of a written, blind, back-translation. In the total sample, 33 interviews were rated by two independent assessors to determine high inter-rater reliability. Prior to the interviews we sent a written informed consent form to all parents or caregivers of the children from class 2 to 7 (age: 6 – 15) explaining the purpose of the study.

Based on these structured interviews, we selected children who had been exposed to high levels of physical and emotional abuse in their homes and those who had been exposed to only low levels of physical and emotional abuse. An *a priori* power analysis (α =.05, power =.80, *d* = .80) using G*Power software (Faul, Erdfelder, Lang, & Buchner, 2007) indicated a required sample size of n = 26 per group to detect significant group differences. Therefore, we aimed for two groups from the extreme ends of the abuse continuum (no abuse vs. high levels of abuse) of 30 children each. As many children, particularly younger children, reported a strong fear of drawing blood, due to harmful experiences in the Tanzanian health system, we decided not to include children of 8 years or younger. We sent an invitation and informed consent form to 96 parents and caregivers of the selected children clarifying that donating blood and saliva samples would be entirely voluntary and no monetary compensation would be offered. In total, 64% (n = 61 of 96) of the parents and caregivers signed the informed consent. We were unable to recruit enough children who had never been exposed to any type of abuse. This is not too surprising, given that several acts of child abuse are culturally normed and highly prevalent in Tanzania. In fact almost 75% reported exposure to physical abuse in a nationally representative sample (UNICEF, 2011). Nevertheless, our sampling approach resulted in two extreme groups; one group (n = 35) reporting high levels of child abuse (i.e., 6 or more different types) and one group (n = 25) reporting low levels of child abuse (i.e., 4 or less different types). In the total sample, 173 (42%) children reported low levels of child abuse with only 8 (2%) reporting no exposure to any form of child abuse. On the other hand, 175 (43%) children of the total sample reported high levels of child abuse. Only children with an informed consent signed by their caregivers and who also assented themselves orally were included in the study (only one child refused to participate despite parents informed consent). A trained nurse from the University of Konstanz with extensive work experience in East Africa collected the blood and saliva samples. The Ethical Review Board of the University of Konstanz approved the study. Other, nonepigenomic parts of the

data gathered for the total sample are presented in reports by Hecker et al. (2014), Hermenau et al. (2014), and Hermenau, Eggert, Landolt and Hecker (2015).

Participants

The children participating in this study were enrolled at a primary school in a city of approximately 150,000 inhabitants in southern Tanzania. The high exposure group consisted of n = 35 children (60% girls) who were on average M = 11.31 years old (SD = 1.47; range: 9 – 15). The low exposure group consisted of n = 25 children (56% girls) who were on average M = 11.76 years old (SD = 1.20; range: 10 – 14).

Measures

All instruments were applied as a structured interview in Swahili. The first part of the interview consisted of socio-demographic information, including age, grade and gender.

Child abuse: We assessed exposure to abuse at home using the Maltreatment and Abuse Chronology of Exposure - Pediatric Version (pediMACE; Isele et al., 2015; Teicher & Parigger, 2015). The pediMACE is a structured interview for children consisting of 45 dichotomous (yes/no) questions, measuring witnessed or self-experienced forms of child maltreatment throughout the lifetime. In this study, we only used the 14 items covering possible forms of physical and emotional abuse (see Table 1) by an adult person living in the same household (e.g. parent, relative or caregiver) or by a minor living in the same household (e.g. housemaid or sibling). In Tanzania many children not only grow up with their parents in one household, but also with other members of their extended families. We also focused on minors in the household as in urban Tanzania many children are raised by an under-aged housemaid (12-17) as primary caregiver, while both parents have to work. Using an event checklist we assessed the presence of different types of abuse but not the frequency. We calculated an abuse score by totaling up all of the question responses. The possible score

ranges from 0 – 14. Cohen's Kappa coefficient measuring the inter-rater reliability was > .99 (.99 – 1).

Mental health: The self-evaluation of internalizing and externalizing problems was assessed with the Strengths and Difficulties Questionnaire (SDQ; Goodman, Ford, Simmons, Gatward, & Meltzer, 2000; Goodman et al., 1998). We used the self-report version for children in interview form, which consists of 25 statements. The total difficulties score is generated by summing the scores of all items, except the items for prosocial behavior, and ranges from 0 to 40. A score over 16 indicates an enhanced level of internalizing and externalizing problems. In the present sample the Cronbach's Alpha coefficient was .71 and the Cohen's Kappa coefficient was .99 (.94 – 1).

The UCLA PTSD Index for Children DSM-IV (Steinberg, Brymer, Decker, & Pynoos, 2004) was used to screen for symptoms of PTSD, again in interview form. For each DSM-IV symptom, the frequency of occurrence within the last month is scored. The PTSD severity score ranges from 0 - 68. In the present sample Cronbach's Alpha was .92 and the Cohen's Kappa .98 (.82 – 1).

The severity of depressive symptoms was assessed by means of the Children's Depression Inventory (CDI; Kovacs, 2001; Sitarenios & Kovacs, 1999), which has already been successfully implemented and validated in Tanzanian settings (Traube, Dukay, Kaaya, Reyes, & Mellins, 2010; Wallis & Dukay, 2009). For each of its 27 items, the children were offered three statements and asked to choose the one which best describes their situation. The maximum score possible is 54. A threshold of 12 has been established as being ideal for identifying children at risk of depression (Kovacs, Goldstein, & Gastonis, 1993; Kovacs, 2001; Traube et al., 2010). In the present sample the Cronbach's Alpha was .81 and the Cohen's Kappa was .99 (.92 – 1).

DNA Methylation: Lymphocytes from blood were isolated via a Ficoll gradient and stored in a preservation solution (DNAgard® Tissues & Cells, Biomatrica, San Diego, USA)

in order to ensure recovery of high quality DNA. In addition, saliva samples were collected and stored using the Oragene•DISCOVER (OGR-500) saliva collection kit (DNA Genotek Inc., Ontario, Canada). The tissue samples were subjected to DNA-extraction (DNeasy® Blood & Tissue Kit, Qiagen, Hilden, Germany). Genome-wide analysis of DNA methylation was then conducted at the Barts and the London Genome Centre (Queen Mary University of London, London, United Kingdom). 1µg of genomic DNA was bisulfite converted (EZ DNA Methylation Kit, Zymo) and applied to the Human Methylation 450K array (Illumina). The raw data were preprocessed using both the R package lumi (Du, Kibbe, & Lin, 2008) and Beta Mixture Quantile Dilation as suggested elsewhere (Marabita et al., 2013). After preprocessing, DNA methylation was assessed for all of the 41, 26, 14 and 14 CpG sites associated with the *GR* gene (*NR3C1*), the *POMC* gene, the *CRH* gene or the *AVP* gene, respectively.

Transcription Factor Binding Sites: To reveal potential functional properties associated with the CpG sites included in our study, the respective sequences were submitted to the Jaspar database (Mathelier et al., 2014) in order to predict known transcription factor binding sites (TFBSs). A conservative threshold of 90% sequence identity was applied.

Data analysis

For the analyses regarding either mental health or exposure to abuse, parametric Welch's t-tests were performed. For DNA methylation, individual 2 (abuse) X 2 (gender) ANOVAs for each CpG site were performed using exposure to abuse and gender as between group factors. We included gender in these analyses in order to account for potential effects arising from gender on DNA methylation. For three CpGs in blood (cg27107893, cg02079741, cg09916783) and one in saliva (cg23035419), the models did not fulfill the requirement of homogeneity of variances, as indicated by a significant Levene's test (Fox & Weisberg, 2011) and are thus not reported. Non-parametric tests could not be performed as these would

not control for the potential influence of gender. In addition, we computed individual 2 (tissue) X 2 (gender) ANOVAs for each CpG site using tissue and gender as between group factors. Due to heterogeneity of variances, 25 probes were excluded from the analyses (*NR3C1*: cg06613263, cg08818984, cg08845721, cg10847032, cg18998365, cg19457823, cg26720913, cg27107893; *POMC:* cg02079741, cg03560973, cg08030082, cg09527270, cg09672383, cg09916783, cg13025668, cg16302441, cg20387815, cg20807790; *CRH*: cg00603617 cg23027580; AVP: cg03279206, cg04360210, cg14065127, cg23035419, cg24257309). Non-parametric tests could not be performed as these would not control for the potential influence of gender.

All analyses used a two-tailed $\alpha = .05$. Our metric for a small effect size was $d \geq .20$ or $\eta^2 \geq .01$, for a medium effect $d \geq .50$ or $\eta^2 \geq .06$, and for a large effect $d \geq .80$ or $\eta^2 \geq .13$. To adjust for multiple testing (for three mental health variables and across the CpG-sites for each gene), p-values were computed according to Benjamin-Hochberg (Benjamini & Hochberg, 1995) applying a false discovery rate of 0.05. In an exploratory approach, we also considered the unadjusted p-values. All statistical analyses were performed using IBM SPSS Statistics version 21 for Mac or R for Mac version 3.0.3.

Results

Mental health

Table 2 displays the descriptive statistics for both groups. In concordance with the sample selection, the high exposure group reported a substantially higher number of different abuse types than the low exposure group. The differences between the two groups are especially notable for the items indicating that a minor in the household was the perpetrator of the abuse (see Table 1). All mental health variables (SDQ, UCLA, CDI) differed significantly between groups with medium to large effects (see Table 2). In total, $n = 11$ (31%) children in the high exposure group showed an enhanced level of internalizing and externalizing problems

compared to *n* = 1 (4%) in the low exposure group. Accordingly, *n* = 9 (26%) children in the high exposure group fulfilled the clinical diagnosis for PTSD compared to *n* = 2 (8%) in the low exposure group. Additionally, *n* = 10 (29%) children in the high exposure group were at risk of suffering from depression compared to *n* = 1 (4%) in the low exposure group.

DNA-methylation of genes associated with the HPA axis

We found a group difference between the high exposure and low exposure group in *POMC* with higher DNA methylation in children with high exposure. This effect was particularly evident in saliva. In the saliva of the high exposure group, one CpG site was significantly hypermethylated in one-tailed tests at an adjusted significance level of .05 and three additional CpG sites would be significantly hypermethylated in one-tailed tests at an adjusted significance level of .10 (Fig. 1, Fig. 2, Table 3). Considering unadjusted p-values as well, three additional CpG sites belonging to *POMC* were differentially methylated in the saliva of the high exposure group. All of the aforementioned CpG sites displayed medium to large effect sizes. In saliva, two more CpG sites in *POMC* displayed moderate effect sizes, although unadjusted p-values exceeded the significance level of .05. In blood, six CpG sites in *POMC* were differentially methylated if unadjusted p-values are considered. These six CpG sites displayed medium to large effect sizes.

For the remaining HPA axis genes investigated we did not find a clear group difference in DNA methylation. In saliva, four CpG sites were differently methylated in *GR* and one in *CRH* displaying moderate effect sizes and unadjusted p-values below .05. In the blood of the high exposure group, one CpG was hypermethylated in *CRH* at an adjusted significance level of .05 displaying a large effect. If uncorrected p-values were considered, one additional differentially methylated CpG could be found in *AVP* displaying a medium effect. If only effect sizes were considered, two additional CpG sites associated with *GR*

differed between the groups in blood displaying moderate effect sizes, but no significant p-values were obtained.

As we found the most pronounced effects in *POMC*, we inspected the seven and nine CpGs, which differed between the groups with at least moderate effect size in blood and saliva, respectively, in more detail. A comparison with the Jaspar-database (Fox & Weisberg, 2011) revealed that five and six of these CpGs are either located in or directly flanking a potential transcription factor binding site (TFBS). The potential TFBSs included TFAP2A, ZEB1, THAP1, YY1, BRCA1, E2F, ZNF354C, MZF1 and SPIB. Interestingly, all of these CpGs are located in the 5'promoter, whose methylation status has been shown to modulate transcriptional activity of the *POMC* gene (Newell-Price, King, & Clark, 2001). Our analyses covered eleven and 12 CpGs in this region in blood and saliva, respectively. In blood, one CpG-site was excluded from the analyses due heterogeneity of variances. Thus, about one half of the CpGs in this region differed in their methylation by means of child abuse, and are associated with TFBSs.

DNA-methylation of the *POMC* gene strengthens children's self-reports

Post-hoc we hypothesized that we could replicate, on the molecular level, the group allocation that was originally based on children's self-reports. We performed unsupervised hierarchical clustering on methylation of the 26 CpG sites representing the *POMC* gene using the Euclidean distance metric and the ward clustering method in the *hclust* package in R. To account for the dispersion differences across the methylation of the CpG sites, data were z-standardized prior to cluster analysis. Both in blood and saliva, two distinct clusters reflecting the high exposure and low exposure group could be detected (Fig. 3). In blood, the analysis allocated n = 39 (68%) children into their respective group and in saliva n = 35 (60%). A chi-square test confirmed the significant concordance between the group

allocation based on children's self-report and based on methylation value in blood (χ^2= 5.95, df = 1, p = .015) and showed a trend in saliva (χ^2= 3.49, df = 1, p = .062).

DNA methylation of HPA axis genes

We additionally compared DNA methylation in the four HPA-axis genes between the two tissues. Generally, blood tended to show stronger signals of DNA methylated than saliva (Fig. 2). The only exception was seen in *AVP*, in which the pattern was reversed and saliva was characterized by elevated DNA methylation levels compared to blood. This tendency was also revealed in the ANOVAs, as we found three, eight, eighteen and eleven CpG sites in *AVP*, *CRH*, *POMC* and *NR3C1*, respectively, which displayed differential methylation between the tissues (Supplementary Table 1).

Discussion

Child abuse is known to impair mental health across the entire lifespan (Carr et al., 2013). However, it has been claimed that the effects of specific forms of child abuse are not as harmful when they take place in societies or cultural groups in which such practices are common, socially accepted and legal. Lansford et al. (2005), for example, demonstrated that the relation between corporal punishment and mental health problems varied with the perceived normativeness of corporal punishment in the respective country. We and others have, however, already demonstrated the detrimental effects of child abuse in such societies (Ani & Grantham-McGregor, 1998; Hecker et al., 2014). Concordantly, in the present study children with high exposure to child abuse showed decreased psychological well-being. Furthermore, we demonstrated that this link manifests itself on a molecular level that cannot be manipulated by the subject: child abuse was strongly associated with the methylation of the *POMC* gene in both blood and saliva. To date, research incorporating child abuse and the methylation of HPA axis genes has focused mainly on the *GR* gene (de Kloet et al., 2005;

Labonte et al., 2014; McGowan et al., 2009; Perroud et al., 2011). Little is known about the physiological and phenotypic consequences of *POMC* methylation. The *POMC* gene is characterized by a 5' CpG-islands, located at exon 1 and the promoter region, and a 3'CpG-island more downstream around the intron 2 and exon 3 boundary (Gardiner-Garden & Frommer, 1994). Research investigating various disease traits or stress exposure have mainly reported differential methylation at the 5' CpG-island (Mizoguchi et al., 2007; Muschler et al., 2010; Newell-Price et al., 2001; Stevens et al., 2010), but effects on the 3' CpG island (Kuehnen et al., 2012) have also been reported. In cancer tissue that did not belong to the piturity gland that caused Cushing's syndrome (hypercortisolism), differential *POMC* methylation at the 5' CpG-island and increased ACTH levels were reported, suggesting HPA axis dysregulation, a key feature of many mental diseases (Mizoguchi et al., 2007; Newell-Price et al., 2001). Our research supports these previous findings, as the majority of differentially methylated CpGs in our study were located in the 5' CpG-island. Moreover, the respective CpGs colocate with transcription factor binding sites (TFBS), suggesting transcriptional regulation. These TFBS include an E2F response element, methylation of which has been shown to suppress POMC promoter activity *in vitro* (Newell-Price et al., 2001).

In addition to ACTH, the functionally relevant peptides β-endorphin and α-melanocyte stimulating hormone (αMSH) are cleaved from the prohormone pro-opiomelanocortin. Thus, the possible impairment of other systems than the HPA axis through *POMC* methylation has to be considered. β-endorphin has anti-nociceptive effects that are essential for stress, in particular the fight-flight situations. It also was reported to have rewarding properties and is considered as a factor in stress-related psychiatric disorders (Merenlender-Wagner, Dikshtein, & Yadid, 2009) and drug abuse (Roth-Deri, Green-Sadan, & Yadid, 2008). Indeed, *POMC* methylation was associated with alcohol craving in patients suffering from alcohol dependence (Muschler et al., 2010). Thus differential *POMC* methylation by means

of child abuse, as found in our study, may heighten the risk of the development of abuse-related mental illness (Carr et al., 2013), including drug abuse (Dube et al., 2003). Abuse seems to affect the methylation of the *POMC* gene and may lead to increased emotional and behavioral problems in the children, which then increase the likelihood for further abuse. In short, settings of frequent abuse would generate in a vicious cycle of further abuse and behavioral problems. Due to the nature of our study, it was not possible to test this idea statistically. Future studies using larger samples and ideally longitudinal designs should test this hypothesis empirically. Nevertheless, our findings are congruent with prior findings that child abuse is related to worse child mental health, even in a society in which specific acts of child abuse are common practice.

DNA methylation profiles appear to be tissue-specific (Ollikainen et al., 2010) and several studies indicated a clear separation of samples derived from saliva and blood (Smith et al., 2014; Thompson et al., 2013; Wu et al., 2014). Accordingly, we found significantly different methylation profiles between saliva and blood. Moreover, there was a general trend of hypermethylation in saliva, which has previously been demonstrated. However, despite tissue-specific methylation, we demonstrate that childhood abuse is associated with DNA methylation in both saliva and blood. Thus methylation evoked by adverse experiences seems to be preserved across tissues.

Moreover, parents and caregivers often argue that children tend to over-report the exposure to abuse and the resulting harm. Thus the children's perception of their experiences is often ignored, as children are not regarded as being mature enough to accurately gauge their situation (Qvortrup, Bardy, Sgritta, & Wintersberger, 1994). Hierarchical clustering based on the methylation of *POMC*, however, allocated two-thirds of children into their respective group and a subsequent chi-square test confirmed the significant concordance between the group allocations based on children's self-report and based on methylation value.

Therefore, our results strengthen the credibility of children's self-reports on a molecular level and support the conclusion that children are indeed capable of accurately reporting their exposure to abuse. In the school context of our data assessment, we were unable to include parents' reports for logistical reasons. Furthermore, we deliberately focused on the credibility of children's reports, as their view has been often neglected in research thus far. While it is possible that the inclusion of parents' reports could have further strengthened our findings, previous studies in resource-poor countries cast doubt on the validity of parents' knowledge about their children's suffering (Elbert et al., 2009).

The methodologies employed by our study present some limitations. Our data are correlational in nature and thus cannot prove a causal relationship between child abuse and methylation patterns or decreased psychological well-being. But even if certain methylation patterns might increase the likelihood of child abuse, the data still confirm the credibility of children's subjective reports and with it a wealth of data showing that abused children are more likely to suffer. However, the sample size and our study design using extreme group comparisons limit the generalizability of our findings. In the school context of our data assessment we were unable to include parents' reports for logistical reasons. Therefore, we could not gather information regarding the socio-economic status (SES) of our sample. It remains to be tested whether SES can impact DNA methylation through other pathways than abuse. Furthermore, it has been suggested that probes containing single nucleotide polymorphisms (SNPs) might result in a biased signal (Price et al., 2013). Based on the *1000 Genomes* project's database (The 1000 Genomes Project Consortium, 2012) eight SNPs colocalize with the target sequence of probes associated with *POMC*. However, the majority of those are very rare in African populations with minor allele frequencies (MAF) below 0.2% and are thus considered not relevant to our sample. Excluding one CpGs, whose respective probe contained a SNP in their target sequence at higher MAF (i.e., 1.0%), did not

markedly change the results (data not shown). Furthermore, this SNP was located more than ten base pairs away from its target CpG, which does not seem to evoke biased signals (Price et al., 2013). Therefore, we consider our findings to reflect the epigenome of the participants and not as artifacts of their genotype.

In summary, we provide further evidence that in societies or cultural groups in which many specific acts of child abuse are common, legal, and socially accepted, child abuse is nevertheless detrimental for the psychological well-being of affected children. Our evidence for such a link is strengthened by the inclusion of epigenetic information from both blood and saliva. This is the first study reporting the link between child abuse and modifications of DNA-methylation of *POMC*. Epigenetic modifications provide a promising mechanism through which child abuse could act to influence psychological well-being. In addition, on a molecular level our study strengthens the credibility of children's self-reports evaluating their exposure to abuse. All in all, our findings underscore the need to inform the population at large about the adverse consequences associated with various forms of child abuse, both those societally accepted and those not. This holds especially true in societies in which such practices are commonly employed and generally regarded as effective.

Competing interests

The authors declare that they have no competing interests.

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Table 1

Occurrence of physical and emotional abuse during the children's lifetime

	High exposure % (*n*)	Low exposure % (*n*)
Physical abuse		
1) Has any adult intentionally pinched, slapped, punched or kicked you?	80 (28)	48 (12)
2) Has any adult spanked you with the palm of his/her hand on buttocks, arms or legs?	74 (26)	24 (6)
3) Has any adult spanked you with an object such as a belt, stick, tube, wooden spoon?	89 (31)	60 (15)
4) Has any adult hit you so hard that you were injured?	40 (14)	4 (1)
5) Has any minor intentionally pinched, slapped, punched or kicked you?	74 (26)	24 (6)
6) Has any minor spanked you with the palm of his/her hand on buttocks, arms or legs?	51 (18)	4 (1)
7) Has any minor spanked you with an object such as a belt, stick, tube, wooden spoon?	31 (11)	0 (0)
8) Has any minor hit you so hard that you were injured?	46 (16)	0 (0)

10) Has any adult yelled or screamed at you?	86 (30)	68 (17)
11) Has any adult called you locked you in a dark & narrow place (e.g. basement, closet)?	20 (7)	0 (0)
12) Has any minor called you names or said hurtful things (e.g. fat, ugly, stupid)?	77 (27)	4 (1)
13) Has any minor yelled or screamed at you?	60 (21)	8 (2)
14) Has any minor called you locked you in a dark & narrow place (e.g. basement, closet)?	3 (1)	0 (0)

Note. Adult = person living in the same household (e.g. parent, relative or caregiver); minor = person under the age of 18 living in the same household (e.g. housemaid or sibling).

Table 2

Demographic characteristics of children with high and low exposure to child abu

	High exposure (n = 35)		Low exposure (n = 25)			
	M	*SD*	*M*	*SD*	*t*	*A*
Abuse types	7.80	1.26	2.64	1.29	15.47	
SDQ score	12.31	5.83	7.48	4.83	3.50	
UCLA score	9.77	11.47	2.04	5.04	3.55	
CDI score	9.14	5.59	3.64	3.33	4.76	

Note. *M*: Mean, *SD*: standard deviation, *t*: test statistics based on Welch-*t*-

adjusted p-value based on Welch-*t*-test corrected for alpha-inflation due to multi

Cohen's d effect size.

Table 3

ANOVAs analyzing the effect of childhood abuse on DNA methylation in CpGs associtated with the *AVP*, *POMC*, *NR3C1* and *CRH* gene.

Gene	CpG	Blood				Saliva			
		F	η^2	*p*	*adj. p*	*F*	η^2	*p*	*adj. p*
AVP	cg03279206	5.82	**.09**	**<.05**	>.10	0.22	.00	>.10	>.10
CRH	cg21240762	0.00	.00	>.10	>.10	4.19	**.07**	**<.05**	>.10
CRH	cg23027580	9.29	**.14**	**<.01**	**<.05**	0.25	.00	>.10	>.10
NR3C1	cg04111177	3.56	**.06**	<.10	>.10	0.23	.00	>.10	>.10
NR3C1	cg06521673	0.37	.01	>.10	>.10	4.32	**.07**	**<.05**	>.10
NR3C1	cg07528216	0.03	.00	>.10	>.10	5.53	**.09**	**<.05**	>.10
NR3C1	cg18849621	0.05	.00	>.10	>.10	4.25	**.07**	**<.05**	>.10
NR3C1	cg19457823	1.09	.02	>.10	>.10	4.54	**.08**	**<.05**	>.10
NR3C1	cg26464411	3.68	**.06**	<.10	>.10	0.25	.00	>.10	>.10
POMC	cg00674304	4.88	**.08**	**<.05**	>.10	1.30	.02	>.10	>.10
POMC	cg01926269	8.04	**.13**	**<.01**	>.10	4.49	**.07**	**<.05**	>.10
POMC	cg09916783	*NA*	*NA*	*NA*	*NA*	6.51	**.10**	**<.05**	**<.10**
POMC	cg11894631	0.32	.01	>.10	>.10	7.93	**.13**	**<.01**	**<.10**
POMC	cg13025668	4.55	**.08**	**<.05**	>.10	10.94	**.16**	**<.01**	**<.05**
POMC	cg14170547	4.18	**.07**	**<.05**	>.10	0.71	.01	>.10	>.10
POMC	cg17736230	2.32	.04	>.10	>.10	8.22	**.13**	**<.01**	**<.10**
POMC	cg20387815	7.23	**.12**	**<.01**	>.10	5.65	**.09**	**<.05**	>.10
POMC	cg24425171	4.20	**.07**	**<.05**	>.10	5.28	**.08**	**<.05**	>.10
POMC	cg24718866	0.18	.00	>.10	>.10	3.68	**.06**	<.10	>.10
POMC	cg09916783	NA	NA	NA	NA	6.51	**.10**	<.05	<.10

Note. F: F statistic for abuse; Adj. p: adjusted p-value ; η^2 = eta square effect size; NA = not available;

p-values below .05, *adj. p*-values below .10 and effect sizes above .06 are highlighted in bold; *AVP = arginine-vasopressin gene; CRH = corticotropin-releasing hormone gene; NR3C1 = glucocorticoid receptor gene, POMC = proopiomelanocortin gene*. Only CpGs, which are differentially either in blood or saliva are displayed.

Figure 1. Mean methylation differences in high and low exposure groups.

The effect size and the level of significance are color-coded or depicted by the shape, respectively. *AVP = arginine-vasopressin gene, CRH = corticotropin-releasing hormone gene, NR3C1 = glucocorticoid receptor gene, POMC = proopiomelanocortin gene.*

Figure 2. DNA methylation of HPA axis genes.

Mean methylation of all analyzed CpG sites. CpG sites are ordered according to their genomic location (not drawn to scale). For visual purposes, the data were mean centered. Beneath the scatterplots, the respective CpG sites and their positions in the gene model are displayed. CpG sites, which revealed at least moderate effect sizes comparing the high and low exposure groups are highlighted in black and bold font. *AVP = arginine-vasopressin gene, CRH = corticotropin-releasing hormone gene, NR3C1 = glucocorticoid receptor gene, POMC = proopiomelanocortin gene, se = standard error.*

•: adjusted $p < 0.1$, *: adjusted $p < 0.05$; **: adjusted $p < 0.01$; ***: adjusted $p < 0.001$

black asterisks/ dots depict tissue comparisons, red asterisks/ dots depict comparisons in relation to child abuse in blood, blue asterisks/ dots depict comparisons in relation to child abuse in saliva.

Figure 3. Hierarchical clustering dendrogram.

Based on the methylation of 26 CpGs present in *POMC* a hierarchical cluster analysis has been performed. Two distinct clusters were formed in both blood (a) and saliva (b) significantly replicating the two groups that are based on children's self-reports regarding exposure to child abuse. The parts of the dendrograms highlighted in red represent the clusters containing mainly children exposed to high levels of child abuse while the turquoise highlighted segments denote the clusters containing mainly children with low exposure. The colored boxes next to the final branches denote the exposure to childhood abuse based on the self-reports (red ≙ high exposure, turquoise ≙ low exposure).

Suppl. Table 1

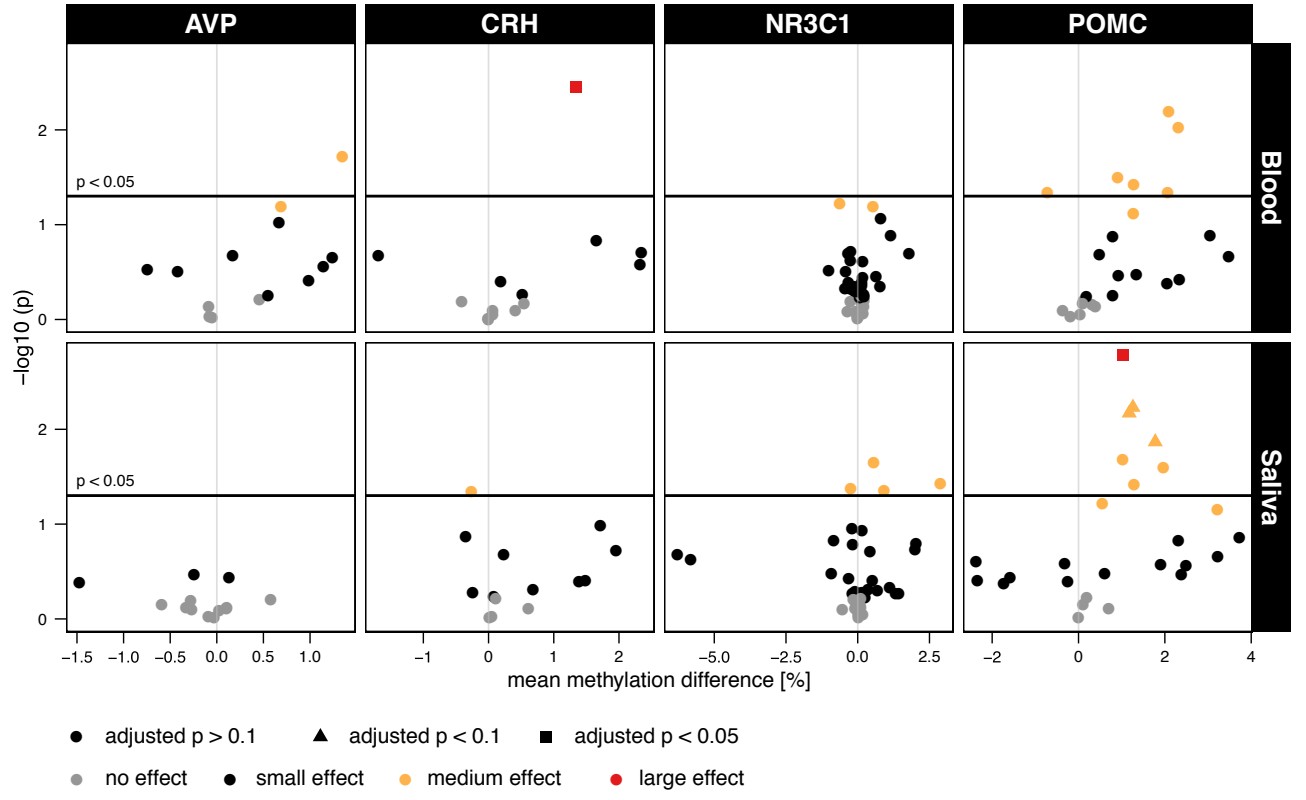
ANOVAs analyzing the effect of tissue on DNA methylation in CpGs associtated with the *AVP*, *POMC*, *NR3C1* and *CRH* gene.

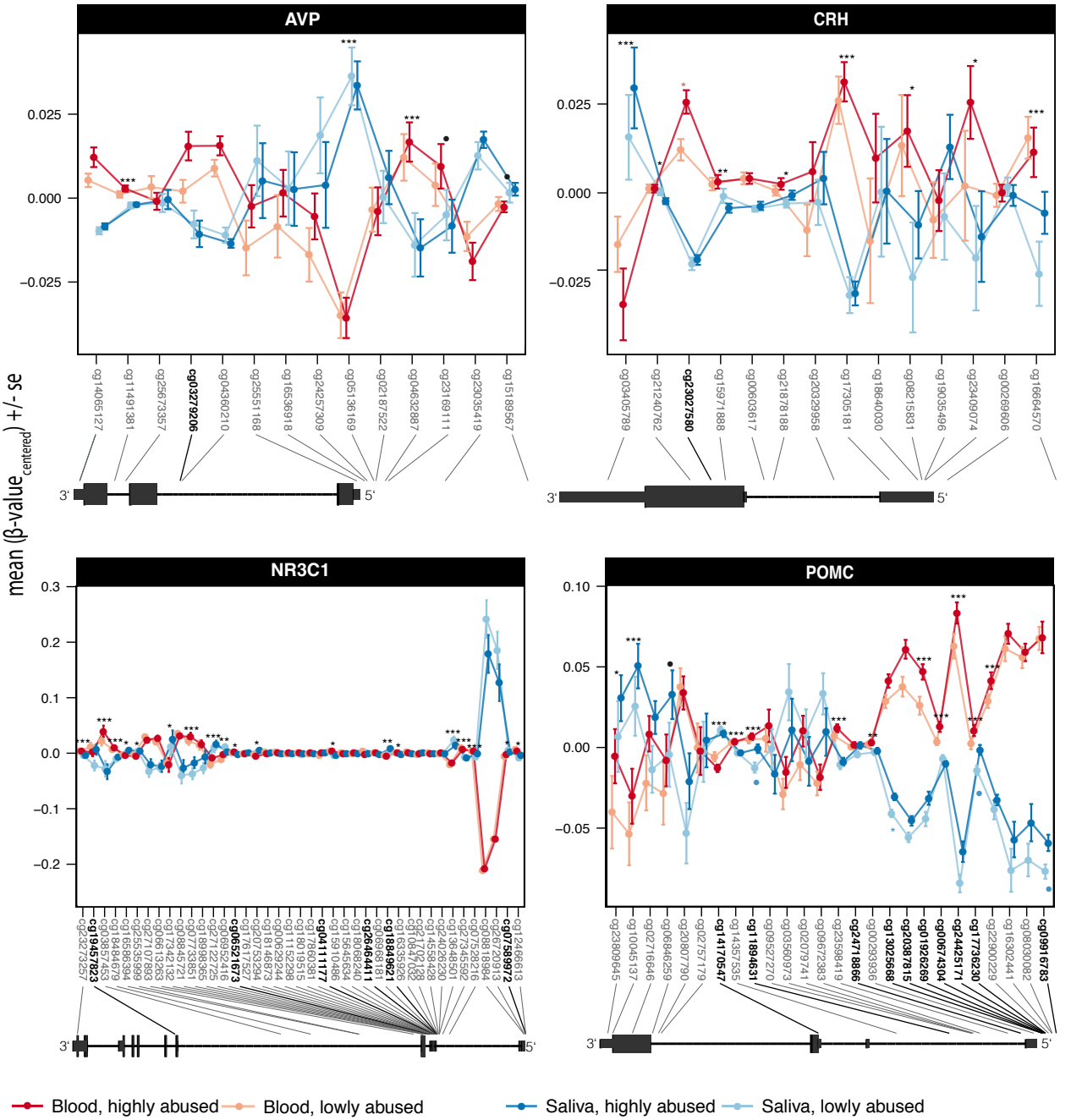
Gene	CpG	*F*	η^2	*p*	*adj. p*
AVP	cg02187522	0.96	0.01	>.10	>.10
AVP	cg04632887	14.25	0.11	<.001	<.001
AVP	cg05136169	99.64	0.46	<.001	<.001
AVP	cg11491381	24.27	0.18	<.001	<.001
AVP	cg15189567	4.47	0.04	<.05	<.10
AVP	cg16536918	0.34	0	>.10	>.10
AVP	cg23169111	3.79	0.03	<.10	<.10
AVP	cg25551168	2.84	0.02	<.10	>.10
AVP	cg25673357	0.38	0	>.10	>.10
CRH	cg00269606	0.05	0	>.10	>.10
CRH	cg03405789	20.17	0.15	<.001	<.001
CRH	cg08215831	7.25	0.06	<.01	<.05
CRH	cg15971888	10.42	0.09	<.01	<.01
CRH	cg16664570	15.16	0.11	<.001	<.001
CRH	cg17305181	127.22	0.53	<.001	<.001
CRH	cg18640030	0	0	>.10	>.10
CRH	cg19035496	0.83	0.01	>.10	>.10
CRH	cg20329958	0.12	0	>.10	>.10
CRH	cg21240762	5.34	0.05	<.05	<.05
CRH	cg21878188	5.53	0.05	<.05	<.05
CRH	cg23409074	5.7	0.05	<.05	<.05

NR3C1	cg00629244	2.59	0.02	>.10	>.10
NR3C1	cg03857453	27.31	0.2	<.001	<.001
NR3C1	cg04111177	0.85	0.01	>.10	>.10
NR3C1	cg06521673	7.67	0.06	<.01	<.05
NR3C1	cg06952416	11.9	0.1	<.001	<.01
NR3C1	cg06968181	0.99	0.01	>.10	>.10
NR3C1	cg07528216	18.28	0.14	<.001	<.001
NR3C1	cg07589972	5.97	0.05	<.05	<.05
NR3C1	cg07733851	34.31	0.23	<.001	<.001
NR3C1	cg11152298	1.24	0.01	>.10	>.10
NR3C1	cg12466613	7.48	0.06	<.01	<.05
NR3C1	cg13648501	41.96	0.27	<.001	<.001
NR3C1	cg14558428	0.04	0	>.10	>.10
NR3C1	cg15645634	0.01	0	>.10	>.10
NR3C1	cg15910486	6.66	0.06	<.05	<.05
NR3C1	cg16335926	7.76	0.06	<.01	<.05
NR3C1	cg16586394	6.01	0.05	<.05	<.05
NR3C1	cg17342132	7.87	0.07	<.01	<.05
NR3C1	cg17617527	0.91	0.01	>.10	>.10
NR3C1	cg17860381	0.66	0	>.10	>.10
NR3C1	cg18019515	3.26	0.03	<.10	>.10
NR3C1	cg18068240	0.72	0.01	>.10	>.10
NR3C1	cg18146873	0.18	0	>.10	>.10
NR3C1	cg18484679	26.71	0.19	<.001	<.001
NR3C1	cg18849621	11.05	0.09	<.01	<.01

NR3C1	cg20753294	6.43	0.05	<.05	<.05
NR3C1	cg21702128	1.74	0.02	>.10	>.10
NR3C1	cg23273257	26.03	0.19	<.001	<.001
NR3C1	cg24026230	0.49	0	>.10	>.10
NR3C1	cg25535999	8.34	0.07	<.01	<.05
NR3C1	cg26464411	0.25	0	>.10	>.10
NR3C1	cg27122725	19.01	0.14	<.001	<.001
NR3C1	cg27345592	53.05	0.32	<.001	<.001
POMC	cg00293936	7.83	0.06	<.01	<.01
POMC	cg00674304	48.82	0.3	<.001	<.001
POMC	cg01926269	230.22	0.67	<.001	<.001
POMC	cg02716646	0.52	0	>.10	>.10
POMC	cg02757179	0.03	0	>.10	>.10
POMC	cg06846259	3.7	0.03	<.10	<.10
POMC	cg10045137	22.19	0.17	<.001	<.001
POMC	cg11894631	19.21	0.14	<.001	<.001
POMC	cg14170547	68.62	0.37	<.001	<.001
POMC	cg14357535	22.22	0.16	<.001	<.001
POMC	cg17736230	16.5	0.13	<.001	<.001
POMC	cg22900229	228.96	0.67	<.001	<.001
POMC	cg23598419	55.59	0.33	<.001	<.001
POMC	cg23809645	4.91	0.04	<.05	<.05
POMC	cg24425171	484.89	0.81	<.001	<.001
POMC	cg24718866	1.47	0.01	>.10	>.10

Note. *F*: F statistic for abuse; *Adj. p*: adjusted p-value; η^2 = eta square effect size; *AVP = arginine-vasopressin gene; CRH = corticotropin-releasing hormone gene; NR3C1 = glucocorticoid receptor gene, POMC = proopiomelanocortin gene.*


AVP
CRH
NR3C1
POMC
Blood
Saliva
p < 0.05
p < 0.05
−log10 (p)
mean methylation difference [%]
adjusted p > 0.1
adjusted p < 0.1
adjusted p < 0.05
no effect
small effect
medium effect
large effect


AVP
CRH
NR3C1
POMC
mean (β-value centered) +/- se
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0.000
−0.025
0.3
0.2
0.1
0.0
−0.1
−0.2
0.10
0.05
0.00
−0.05
3'
5'
cg14065127
cg11491381
cg25673357
cg03279206
cg04360210
cg25551168
cg16536918
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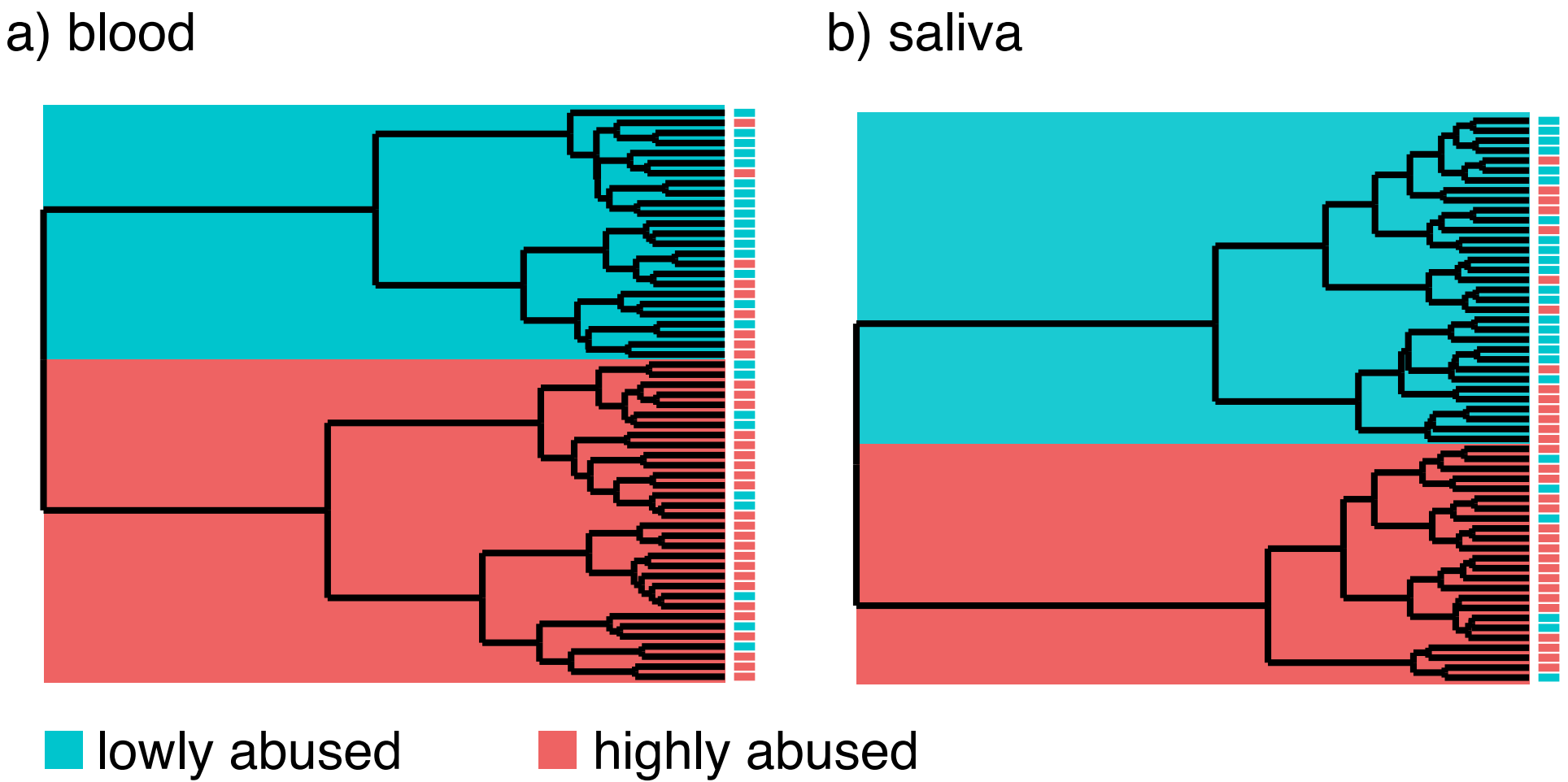
Blood, highly abused
Blood, lowly abused
Saliva, highly abused
Saliva, lowly abused


a) blood
b) saliva
lowly abused
highly abused